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Enzymatic regulation of functional vascular networks using gelatin hydrogels

Chia-Hui Chuang^a, Ruei-Zeng Lin^b, Han-Wen Tien^a, Ya-Chun Chu^a, Yen-Cheng Li^c, Juan M. Melero-Martin^b, and Ying-Chieh Chen^{a,*}

^aDepartment of Applied Science, National Hsinchu University of Education, Hsinchu 30014, Taiwan, ROC

^bDepartment of Cardiac Surgery, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA

^cMaterial and Chemical Research Laboratories, Industrial Technology Research Institute, Hsinchu 31040, Taiwan, ROC

Abstract

To manufacture tissue engineering-based functional tissues, scaffold materials that can be sufficiently vascularized to mimic the functionality and complexity of native tissues are needed. Currently, vascular network bioengineering is largely carried out using natural hydrogels as embedding scaffolds, but most natural hydrogels have poor mechanical stability and durability, factors that critically limit their widespread use. In this study, we examined the suitability of gelatin-phenolic hydroxyl (gelatin-Ph) hydrogels that can be enzymatically crosslinked, allowing tuning of the storage modulus and the proteolytic degradation rate, for use as injectable hydrogels to support the human progenitor cell-based formation of a stable and mature vascular network. Porcine gelatin-Ph hydrogels were found to be cytocompatible with human blood-derived endothelial colony-forming cells and white adipose tissue-derived mesenchymal stem cells, resulting in >87% viability, and cell proliferation and spreading could be modulated by using hydrogels with different proteolytic degradability and stiffness. In addition, gelatin was extracted from mouse dermis and murine gelatin-Ph hydrogels were prepared. Importantly, implantation of human cell-laden porcine or murine gelatin-Ph hydrogels into immunodeficient mice resulted in the rapid formation of functional anastomoses between the bioengineered human vascular network and the mouse vasculature. Furthermore, the degree of enzymatic crosslinking of the gelatin-Ph hydrogels could be used to modulate cell behavior and the extent of vascular network formation *in vivo*. Our report details a technique for the synthesis of gelatin-Ph hydrogels from allogeneic or xenogeneic dermal skin and suggests that these hydrogels can be used for biomedical applications that require the formation of microvascular networks, including the development of complex engineered tissues.

*Corresponding author at: Department of Applied Science, National Hsinchu University of Education, No. 521, Nanda Rd., Hsinchu 30014, Taiwan, ROC. Tel.: +886 3 5213132 2763; fax: +886 3 5257178. yingchiehchen@gmail.com (Y.-C. Chen).

Keywords

Gelatin hydrogels; Vascularization; Tissue engineering; In vivo; Vascular graft

1. Introduction

Although there have been extensive advances in the creation of vascularized tissues constructs, most successes in tissue engineering have been limited to avascular or thin tissues *in vivo* [1,2]. The major hurdle in the development of more complex tissues is the ability to generate functional vascular networks in three-dimensional (3D) environments. Bioengineered vascular networks need to be generated within clinically suitable biomaterials and to be able to develop rapidly to ensure complete vascularization of embedded cells and the ingrowth of pre-existing host microvessels to avoid necrosis [3–5]. Thus, the search for a suitable biomaterial that can serve as a scaffold for the cells is very important.

Recently, several studies [6–10] have shown that endothelial colony-forming cells (ECFCs) have vasculogenic potential that can be exploited to generate long-lasting and stable vascular networks *in vivo*. Importantly, these studies have also shown that, to generate stable and durable vascular networks, ECFCs require co-implantation of mesenchymal stem cells (MSCs) or fibroblasts as perivascular cells [7,11–13]. In most current models of vasculature formation, cells are embedded in Matrigel or other natural collagen-based hydrogels that are favorable microenvironments for vascular morphogenesis [7,10,14–16]. However, some properties of these materials are not ideal for tissue engineering applications, as Matrigel is a gelatinous protein mixture secreted by murine sarcoma cells and therefore not suitable for clinical use [17], while collagen-based gels have a weak bulk mechanical strength and low modulus and exhibit significant contraction after being mixed with cells [6,18,19]. Furthermore, the clinical use of animal-derived extracellular matrix (ECM) proteins is often restricted because of immunogenic concerns. Accordingly, improving the mechanical strength and durability of naturally occurring ECMs and overcoming possible immunogenicity concerns are crucial priorities in tissue engineering research on a broad range of various tissues/organs, and these aims can be achieved by the chemical functionalization of proteins [20]. A recent example of material functionalization is the development of photocrosslinkable methacrylated gelatin (GelMA) hydrogels, which are synthesized by adding methacrylate groups to the amine-containing sidegroups of gelatin [21,22]. Recently, we demonstrated that a hydrogel of this type is fully compatible with ECFC-based vascular morphogenesis and proposed its use for vascular bioengineering applications [13,23]. In addition, we demonstrated that GelMA can polymerize both *ex vivo* and *in vivo* within 15 s upon exposure to UV light in the presence of a photoinitiator, and that such gels can be used to deliver vascular cells in regenerative applications that require the formation of functional vascular beds *in vivo* [13,23]. However, UV irradiation is only effective for thin and/or transparent scaffolds and materials that allow the passage of UV light. Another type of hydrogel of interest is enzymatically cross-linked hydrogels, generated by mild reaction conditions. The majority of the enzymes involved in the crosslinking are enzymes catalyzing reactions naturally occurring in the body. Enzymatic reactions are catalyzed by most enzymes at neutral pH, in aqueous solution, and at a

moderate temperature, and these mild conditions might also be used to develop hydrogels that form in situ [24]. Additionally, unwanted side reactions or toxicity that can occur with the use of photo-initiators or organic solvents are avoided. The polymerization reaction can be directly controlled by the modulation of enzyme activity and concentration. One recent example is the development of gelatin-phenolic hydroxyl (gelatin-Ph) hydrogels [25–28], which are synthesized by adding the phenolic hydroxyl groups of tyramine to the carboxyl side-groups of gelatin to form a gelatin-Ph conjugate and enzymatically cross-linking this conjugate. As a result, gelatin-Ph hydrogels combine certain advantages of both natural and synthetic biomaterials. In particular, they contain gelatin as the backbone, which provides cell adhesion sites and proteolytic degradability. Moreover, it is possible to tune the mechanical and chemical properties of the hydrogels by modifying the level of phenolic hydroxyl (Ph) group conjugation and enzymatic crosslinking to create 3D microarchitectures [27–29].

In this paper, we report the synthesis and characterization of various hydrogels formed from porcine gelatin-Ph conjugates by HRP-/H₂O₂-induced crosslinking via C–C or C–O bonds between the tyramine moieties added to the gelatin. The mechanical properties, swelling behavior, and proteolytic degradability of these hydrogels were investigated. We also studied the growth of adherent human blood-derived ECFCs and white adipose tissue-derived MSCs on, or in the hydrogels. Due to concerns about pathogen transmission between species and immunogenic concerns about the use of ECM proteins from a different species, autologous murine gelatin-Ph hydrogels were also developed and evaluated for use in immunodeficient mice. Finally, in order to examine the feasibility of the formation of functional vascular networks *in vivo*, a liquid prepolymer solution of gelatin-Ph containing human blood-derived ECFCs and white adipose tissue-derived MSCs, HRP, and H₂O₂ was injected into the subcutaneous space of an immunodeficient mouse before gelation and was demonstrated to form a 3D cell-laden polymerized construct in the mouse. These results show that extensive human ECFC-lined vascular networks can be generated, that the final vascular density inside gelatin-Ph hydrogel constructs can be manipulated through tunable mechanical properties and proteolytic degradability, and that these networks can form functional anastomoses with the existing vasculature in a host mouse.

2. Materials and methods

2.1. Synthesis and characterization of porcine gelatin-Ph conjugates

Gelatin-Ph conjugates were synthesized by combining gelatin and tyramine hydrochloride by carbodiimide-mediated condensation of the carboxyl groups of gelatin and the amino groups of tyramine, resulting in the binding of a Ph group to the carboxyl group. Two g of gelatin powder (type A from porcine skin, Sigma Aldrich) was dissolved in 100 ml of 50 mM morpholinoethanesul-fonic acid (MES, Sigma Aldrich) by heating at 60 °C for 30 min, then the solution was cooled to 27 °C and the pH adjusted to the required value using 4 N NaOH or 4 N HCl. Tyramine (1 g) was then added and the carboxylic acid groups of gelatin activated by adding 0.735 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.221 g of N-hydroxysulfosuccinimide (NHS) and allowing the mixture to react for different time periods (up to 48 h) and at different temperatures (17, 37,

and 47 °C) and pH values (3–8) to synthesize gelatin-Ph conjugates (step 1), then the reaction was stopped by adding 0.82 g of sodium phosphate to increase the pH above 9 for 30 min, then the resultant polymer solution was dialyzed against deionized water using an ultrafiltration membrane (MWCO: 3500) until no absorbance peak at 275 nm due to residual tyramine was detectable in the filtered solution. The conjugation of Ph groups to porcine gelatin under these different conditions was investigated by measuring gelation time in step 2 and conjugate yield. In order to optimize conditions for chemical immobilization of tyramine on gelatin, surface zeta-potential analysis (Malvern Zeta Nanosizer) was performed on porcine gelatin solutions dissolved in different buffers.

In further studies, two combinations of different concentrations of EDC and NHS (0.735 g of EDC and 0.221 g of NHS or 0.147 g of EDC and 0.044 g of NHS) were added to 100 ml of 2% porcine gelatin in 50 mM MES buffer, pH 6, and reaction was carried out at 27 °C for 12 h, generating conjugates with, respectively, a higher or lower tyramine content, designated as gelatin high-Ph and gelatin low-Ph conjugates, which were then dialyzed against deionized water and lyophilized. The conjugates were then dissolved at 5 mg per ml in D₂O, and proton nuclear magnetic resonance (¹H NMR) spectra were recorded on a Bruker AV-400 (400 MHz) spectrometer at room temperature to obtain an indication of how much tyramine was immobilized in the gelatin low-Ph and gelatin high-Ph conjugates. The exact amount of tyramine bound to gelatin was quantified by dissolving the tyramine-substituted gelatin as a 0.1% (w/w) solution in distilled water and measuring the absorbance at 275 nm on an ultraviolet-visible spectrometer and estimating the content of introduced Ph groups using a calibration curve of different concentrations of tyramine hydrochloride in distilled water.

2.2. Hydrogel formation and gelation time

For hydrogel formation, freshly prepared solutions of horseradish peroxidase (HRP) and hydrogen peroxide (H₂O₂) (10 µl of each) (both from Sigma-Aldrich) at the indicated concentrations in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (Ca/Mg-free DPBS) were added to 80 µl of 12.5% (w/v) gelatin-Ph in Ca/Mg-free DPBS and mixed (step 2), then the solution was examined for formation of the hydrogel state (starting to go viscous) and the end of gelation (formation of a definite shape).

2.3. Hydrogel characterization

A hemolysis assay was performed by adding 100 µl of whole rabbit blood to 10 ml of 1% (w/v) porcine gelatin-Ph in PBS and incubating the mixture at 37 °C with mild shaking for 1 h. The samples were then centrifuged at 2000 g for 5 min and the hemoglobin concentration in the supernatant measured by the absorbance at 550 nm using UV absorption spectroscopy. A negative control of phosphate-buffered saline (PBS) and a positive control of distilled water were also used for comparison.

Rheological characteristics were examined using a rheometer (AR-G2, TA Instruments) operating with parallel plate geometry (25 mm diameter plates) at 37 °C in oscillatory mode. For each measurement, porcine gelatin-Ph hydrogels formed using two different concentrations of HRP and H₂O₂ (12.5 units/ml of HRP plus 0.31–80 mM H₂O₂ or 0.078

units/ml of HRP plus 0.31–10 mM H₂O₂) were applied to the bottom plate and the upper plate was lowered to form a measurement gap of 1.4 mm. Two hours later, a frequency of 10 rad/s and a strain of 1% were used in the analysis to maintain the linear viscoelastic behavior and measure the storage modulus; measurement was continued until the recorded storage modulus reached a plateau.

For swelling tests, gelatin hydrogels, prepared as described above, were immersed overnight at room temperature in 1 ml of PBS for 24 h at 37 °C, then the swollen hydrogels were weighed and lyophilized. The swelling ratio was defined as the amount of water absorption (swollen mass minus dried mass) divided by the dried mass (after lyophilization).

The enzymatic degradation properties of porcine gelatin hydrogels with two different degrees of cross-linking (high-Ph and low-Ph) were determined at 37 °C in PBS containing 2 U/ml of collagenase type I (Sigma Aldrich), the percentage mass loss being determined at different time points as 100× the original weight minus the final weight divided by the original weight.

2.4. Cell culture

ECFCs and MSCs were isolated, respectively, from human cord blood and white adipose tissue, as described previously [10,30–32]. ECFCs were cultured on rat tail collagen type-I (5 µg/cm², BD)-coated tissue culture plates in endothelial basal medium (Lonza) supplemented with 20% fetal bovine serum (FBS) (Hyclone), SingleQuots (containing human epidermal growth factor, human recombinant fibroblast growth factor-β, vascular endothelial growth factor, insulin-like growth factor, ascorbic acid, heparin, and gentamicin/amphotericin-B; Lonza), and 1 × penicillin-streptomycin (PS) (Invitrogen). MSCs were cultured on uncoated plates using mesenchymal stem cell growth medium (Lonza) containing 10% FBS, 1 × PS, and 10 ng/ml of basic fibroblast growth factor (Peprotech). ECFCs and MSCs between passages 8 to 10 were used in all experiments.

NIH-3T3 cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Hyclone) and 1 × PS at 37 °C and in 5% CO₂.

2.5. Cell attachment, viability, proliferation, and spreading assays on porcine gelatin low-Ph hydrogels

Eight volumes of porcine gelatin low-Ph conjugates dissolved in Ca/Mg-free DPBS at 12.5% (w/v) were mixed with 1 volume each of concentrated HRP or H₂O₂ solution, resulting in a final concentration of 10% (w/v) gelatin low-Ph conjugate, 0.078 units/ml of HRP, and 0.31–2.5 mM H₂O₂ in Ca/Mg-free DPBS, and the mixture immediately poured into each well (300 µl/well) of a 48-well plate, and the hydrogels allowed to form for 2 h at room temperature. Unmodified porcine gelatin-coated dishes were used as a control substrate; 300 µl of 1% porcine gelatin solution was added to each well of a 48-well cell culture plate, which was then incubated for 30 min at 37 °C, and non-bound gelatin removed by washing the wells with PBS.

The effects of porcine gelatin hydrogels cross-linked with different H_2O_2 concentrations (0.31–2.5 mM) on cell adhesion, proliferation, viability, and spreading were determined at 37 °C and in 5% CO_2 as follows. To examine attachment, ECFCs or MSCs were seeded at a density of $1 \times 10^4/\text{cm}^2$ on hydrogel or gelatin-coated wells and incubated for 6 h, while, to examine proliferation, they were seeded at a density of $7.5 \times 10^3/\text{cm}^2$ and incubated for 24 h, then the number of attached or proliferated cells was assessed by MTS assay (Sigma–Aldrich) following the manufacturer’s instructions. The viability of cells on the gels was determined using Live/Dead staining kits (Invitrogen) by imaging green-stained cells (live cells) and red-stained cells (dead cells) under a fluorescent microscope and counting and analyzing cells in five randomly selected fields of view under 200× magnification using ImageJ software and dividing the number of live cells by the total number of cells. The spreading area and length occupied by each cell was measured using ImageJ software.

2.6. Enzymatic and oxidative stress challenge assays

The effects of these two stress factors were examined using mouse NIH-3T3 fibroblasts embedded in 10% (w/v) porcine gelatin high-Ph hydrogels crosslinked using 0.078 units/ml of HRP and 1.25 mM H_2O_2 or cultured on top of, or under, the same hydrogel, then NIH-3T3 culture medium containing 0–10 units/ml of HRP or 0–100 μM H_2O_2 was added for 24 h to induce enzymatic (HRP) or oxidative stress (H_2O_2).

The effect of encapsulation was examined by culturing cells encapsulated at $4 \times 10^6/\text{ml}$ in hydrogels for 24 h before stress was applied using HRP or H_2O_2 as detailed above. For culture on hydrogels, cells were seeded at 5×10^4 cells/ cm^2 on top of the gelatin high-Ph hydrogel and cultured for 24 h in NIH-3T3 culture medium containing HRP or H_2O_2 as above; additionally, to evaluate whether gelatin high-Ph conjugates could prevent the loss of viability of cells exposed to oxidative stress, cells seeded on gelatin high-Ph hydrogels were incubated with 10% (w/v) of gelatin high-Ph conjugates in NIH-3T3 culture medium containing 0–100 μM H_2O_2 for 24 h. For culture under hydrogels, cells were seeded at 5×10^4 cells/ cm^2 into each well in a 24-well plate and cultured for 4 h, when 300 μl of the hydrogel was added to each well to cover the cell layer, then, after 2 h of culture, NIH-3T3 culture medium containing 0–100 μM H_2O_2 was added for 24 h to induce oxidative stress. The number of surviving cells was then measured using MTS (Sigma–Aldrich) according to the manufacturer’s instructions. In all tests, background absorbance, measured using cell-free hydrogels, was subtracted prior to analysis.

2.7. Extraction of murine gelatin from the epidermal tissue of nude mice and synthesis of murine gelatin–Ph hydrogels

Before gelatin extraction, mouse epidermal tissue was cut into small pieces ($<25 \text{ mm}^2$) and soaked for 24 h at room temperature in 3% acetic acid at a skin/solution ratio of 1:20 (w/v), then the mixture was stirred continuously overnight at room temperature to swell the collagenous material in the skin matrix. The acid-treated skin was then washed with tap water until the pH of the wash water was neutral. To extract gelatin, the swollen skin was soaked for 10 min at room temperature in distilled water at a skin/water ratio of 1:30 (w/v), then the mixture was autoclaved for 30 min at 121 °C, sequentially filtered using Whatman No. 1 filter paper and filter membranes with a pore size of 0.45 μm , and the filtrate freeze-

dried. The gelatin samples were weighed and the extraction yield calculated, then the samples were subjected to SDS polyacrylamide gel electrophoresis, measurement of hydroxyproline (hyp) content [33], and amino acid analysis (Waters, Model Workstation, USA), and tested for effects on cell function.

Murine gelatin-Ph conjugates were synthesized essentially in the same way as porcine gelatin-Ph conjugates. Freeze-dried murine gelatin (2 g) was dissolved in 100 ml of 50 mM MES, pH 6, by heating to 60 °C, then murine gelatin-Ph conjugates were prepared by the addition of 0.147 g of EDC and 0.044 g of NHS, followed by 1 g of tyramine, incubating the mixture for 12 h at 27 °C, and stopping the reaction by the addition of sodium phosphate (0.82 g) to increase the pH above 9 for 30 min. The resultant polymer solution was dialyzed against deionized water using an ultrafiltration membrane (MWCO: 3500) until no residual tyramine was detected in the filtered solution.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Porcine or murine gelatin was dissolved in 1 × Laemmli sample buffer (Bio-Rad Laboratories) containing 2.5% 2-mercaptoethanol and 1 × protease inhibitors (Thermo) and heated at 95 °C for 5 min, then samples (30 µg total protein) were loaded onto a 10% SDS polyacrylamide gel. SDS-PAGE was performed using a Mini Protein II unit (Bio-Rad Laboratories) at a constant voltage of 100 V, then protein bands were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) and the gels destained following the manufacturer's instructions.

2.9. In vivo vasculogenic assay

The formation of vascular networks *in vivo* was evaluated using a xenograft model of transplantation into immunodeficient mice [7,32,34]. Six-week-old male BALB/cAnN.Cg-Foxnlnu/CrlNarl nude mice were purchased from the National Laboratory Animal Center, Taiwan. All procedures involving animals and their care were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Vasculogenesis was evaluated *in vivo* using our xenograft model as described previously [7,32,34]. Briefly, for porcine gelatin-Ph hydrogels, ECFCs and MSCs (2×10^6 total; 2:3 ECFC/MS ratio) were suspended in 250 µl of 12.5% (w/v) porcine gelatin-Ph, then concentrated H₂O₂ and HRP were added to give final concentrations of 10% porcine gelatin-Ph, 0.078 units/ml of HRP, and 0.31–2.5 mM H₂O₂. The mixture was then injected subcutaneously into mice and the hydrogel formed inside the mouse within 3 min. Murine gelatin-Ph hydrogels used in animal studies were at final concentration of 10% (w/v), 0.078 units/ml of HRP, and 0.63 mM H₂O₂. Rat tail type-I collagen gel (3 mg/ml in PBS, pH 7.4) and phenol red-free Matrigel (both from BD Bioscience, San Jose, CA) containing the same number of cells served as controls. All experiments were carried out 4 times, each on 1 or 2 mice; the value reported is the mean result for the 4 studies.

2.10. Histological and immunohistochemical analysis

At one week after xenografting, the mice were euthanized and the hydrogel implants removed, weighted and fixed overnight at room temperature in buffered formalin (10%, pH 7.2–7.4; Sigma-Aldrich), and embedded in paraffin, then 7 µm sections were prepared.

Hematoxylin and eosin (H&E) staining was used to detect luminal structures containing red blood cells. For immunohistochemistry, the sections were deparaffinized, and antigen retrieval carried out by heating the sections for 10 min at 93 °C in 10 mM Tris-base, 2 mM EDTA, 0.05% Tween-20, pH 8.0. The sections were then blocked by incubation for 30 min at room temperature in blocking buffer (5% BSA in PBS) and incubated for 1 h at room temperature with mouse anti-human CD31 antibodies (1:50 in blocking buffer; DakoCytomation, clone JC70A), mouse anti-human α -smooth muscle actin (α SMA) antibodies (1:200; Sigma–Aldrich, A2547 clone 1A4), or mouse IgG (1:50 in blocking buffer; DakoCytomation). For mouse anti-human CD31 immunohistochemistry, the sections were incubated for 1 h at room temperature with HRP-conjugated mouse secondary antibodies (1:200 in blocking buffer; Vector Laboratories), then bound antibody was detected using a 3,3'-diaminobenzidine detection kit (Vector Laboratories) according to the manufacturer's protocol, followed by hematoxylin counterstaining and Permount mounting. For mouse anti-human CD31 and mouse anti-human α SMA immunofluorescence studies, the sections were incubated for 1 h at room temperature with, respectively, Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:200; Invitrogen). All fluorescently stained sections were counterstained with DAPI (Invitrogen).

2.11. Microvessel density

Microvessels were quantified by evaluation of 10 randomly selected fields ($400 \times$ magnification) of H&E-stained sections taken from the central part of the implant. Microvessels were identified as luminal structures containing red blood cells. Microvessel density was calculated by dividing the total number of red blood cell-containing microvessels by the area of each section and expressed as vessels/mm². The percentage of human microvessels in the total microvessels and the luminal areas of the human microvessels were determined using ImageJ software after human-CD31 immunohistochemical staining. The values reported for each experimental condition are the mean $\pm$ the standard deviation for seven individual mice.

2.12. Statistical analysis

Statistical analysis was performed using Microcal Origin 8.0 (OriginLab Corporation.). All data are presented as the mean $\pm$ standard deviation. Comparison of values for different materials was performed using one-way/two-way ANOVA. Significance levels were set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results and discussion

3.1. Synthesis and characterization of porcine gelatin-Ph conjugates

Given the harmful effects of H₂O₂, a strong oxidant, on encapsulated cells and surrounding tissues *in vivo*, fast gelation at a low H₂O₂ concentration is desirable for practical tissue-engineering applications. We therefore optimized the conjugation conditions to allow rapid hydrogel formation at low H₂O₂ and HRP concentrations.

First, we measured the zeta potential of porcine gelatin dissolved in distilled water or 50 mM MES buffer as a function of pH. The zeta potential is a key indicator of the stability of

the gelatin molecule and helps evaluate the net electrical charge of gelatin in solution, which affects the coupling efficiency and total yield of step 1, with a charge of zero resulting in the best conjugation. As shown in Fig. S1a, the zeta potential in distilled water was +3 to +5 between pH 4 and pH 8, while, in MES, it was $+14.6 \pm 3.3$ mV at pH 4, but decreased rapidly with increasing pH to approximately 0 mV between pH 6 and pH 9.

In the next series of experiments, we evaluated the optimal conditions for forming a gelatin-Ph conjugate that allowed rapid gelation on the addition of HRP and H_2O_2 using 2 g of gelatin, 1 g of tyramine, 0.735 g of EDC, and 0.221 g of NHS in 100 ml of 50 mM MES buffer for the coupling step (step 1), and 10% conjugate and 0.156 units/ml of HRP and 0.63 mM H_2O_2 to form the hydrogel (step 2).

We first examined the effect of the pH used in step 1 at 27 °C for 12 h in 50 mM MES buffers of increasing pH and found that the subsequent gelation time (step 2) decreased from 81 s using conjugates formed at pH 3 to less than 1 s using those formed at pH 7 and 8 (Fig. S1b, black dots). In addition, to measure the yield of gelatin-Ph conjugates from gelatin, defined as the dry weight of gelatin-Ph conjugates divided by the dry weight of gelatin, after step 1, we freeze-dried the gelatin-Ph solutions and found that the yield ranged from 60% to 80% (Fig. S1b, blue dots).

Since the speed of gelation was too rapid at pH 7 and 8 for practical use, we chose to use 50 mM MES buffer, pH 6, in step 1 and examined the effect of different reaction times at 27 °C and found that a reaction time of 12 h gave the shortest gelation time (40 ± 1 s; Fig. S1c, black dots) with a yield of gelatin-Ph conjugates of around 60% (Fig. S1c, blue dots).

Using 50 mM MES buffer, pH 6, and a 12 h reaction time, we then examined the effect of reaction temperature (27, 37, and 47 °C) and found that the shortest gelation time was obtained using conjugates formed at 27 °C (34 ± 4 s) with a yield of $69.8 \pm 9.3\%$ (Fig. S1d).

We then examined the gelation time of conjugates formed at 27 °C for 12 h using the same concentrations in step 1 of gelatin, tyramine, EDC, and NHS as above in 100 ml of deionized water, 50 mM Tris buffer, or 50 mM MES buffer at either pH 6 (Fig. S1e) or 7 (Fig. S1f), but a lower HRP concentration of 0.039 units/ml and a higher H_2O_2 concentration of 1.25 mM in step 2, and found that the shortest gelation time (19 ± 1 s) was observed using conjugates formed in Tris-HCl, pH 7.

In order to compare the degree of Ph group conjugation to gelatin (measured by the gelation time, which decreases with Ph content) obtained using 5% and 10% conjugates in our system with that reported by Sakai's group using 5% conjugates, 50 mM MES buffer, pH 6, and a 12 h reaction time [28], we used gelatin-Ph conjugates formed by incubation of 2 g of gelatin, 1 g of tyramine, 0.735 g of EDC, and 0.221 g of NHS in 100 ml of either 50 mM Tris buffer, pH 7, or 50 mM MES buffer, pH 6, at 27 °C for 12 h in step 1, and incubated the conjugates at final concentrations of 5% (w/v) and 10% (w/v) in Ca/Mg-free DPBS with 1.25 units/ml of HRP and 5 mM H_2O_2 in step 2 to form the hydrogel. Macroscopic images (Fig. S1g) showed that the hydrogels formed using 5% or 10% gelatin-Ph conjugates in Tris buffer, pH 7, and using 10% gelatin-Ph conjugates in MES, pH 6, remained transparent, while those generated using 5% gelatin-Ph conjugates in MES, pH 6, turned white after the

addition of HRP and H_2O_2 . Gelation occurred in 18 ± 2 s using 5% conjugate in Tris, pH 7, 8 ± 1 s using 10% conjugate in Tris, pH 7, 4 ± 1 s using 10% conjugate in MES, pH 6, and 16 ± 1 s using 5% conjugate in MES, pH 6 (Fig. S1h), the last conditions being identical to those used by Sakai's group, who quoted a gelling time of 20 s [28]. However, all of the hydrogels formed in our study using conjugates synthesized in either Tris, pH 7, or MES, pH 6, (Fig. S1g) were more transparent than those reported by Sakai's group. No precipitation was seen at any stage during synthesis and prior to gelation. The total yields in step 1 after conjugation under different conditions ranged from 55% to 83% (Fig. S1b–f, blue dots). In all subsequent studies, we used 10% conjugate in hydrogel formation.

To increase the amount of Ph conjugated to gelatin, in a further study, two different concentrations of 0.735 g of EDC plus 0.221 g of NHS or 0.147 g of EDC plus 0.044 g of NHS were added to 2 g of gelatin in 100 ml of 50 mM MES buffer, pH 6, and reaction was carried out at 27 °C for 12 h. As shown in Fig. S2a, this resulted in the formation of conjugates with, respectively; a higher or lower Ph content as measured by UV–Vis spectrometry, designated gelatin high-Ph and gelatin low-Ph conjugates. The ^1H NMR spectra of both the porcine gelatin low-Ph and high-Ph samples (Fig. 1a) showed peaks at chemical shifts (δ) of 6.8 ppm and 7.1 ppm that were not seen in unmodified porcine gelatin and can be attributed to the presence of phenol groups, but the integrated phenol peaks were higher in the gelatin high-Ph conjugates. These results indicate successful conjugation of Ph to the carboxyl groups of the amino acid residues of gelatin. Furthermore, conjugation of Ph was quantitatively analyzed by measuring the absorbance at 275 nm. The total Ph contents per gram of gelatin using the two preparation conditions shown in Fig. S2a were 5.3×10^{-3} g/g of gelatin (gelatin low-Ph) and 1.2×10^{-2} g/g of gelatin (gelatin high-Ph). As shown in Fig. S2b, the macroscopic images of 10% gels formed using gelatin high-Ph conjugate (left panel) or gelatin low-Ph conjugate (right panel), 0.078 units/ml of HRP, and increasing H_2O_2 concentrations [0.625–5 mM (left panel) or 1.25–5 mM (right panel)] showed no difference in opacity, but, as shown in Fig. S1g, 5% gels formed using gelatin high-Ph conjugate, 1.25 units/ml HRP, and 5 mM H_2O_2 were slightly white.

3.2. Porcine gelatin hydrogel formation and characterization

Chemically cross-linked gels (formed by stronger and stable covalent bonds, including enzymatic or photochemical cross-linked hydrogels) are considered superior to physically cross-linked gels (formed by molecular entanglements, and/or secondary forces including ionic, hydrogen bonding or hydrophobic interactions, such as collagen and Matrigel) as injectable scaffolds because of the controllable gelation time and mechanical and degradation properties. Fig. 1b and c shows the dependence of gelation time on the concentration of HRP and H_2O_2 and the content of Ph groups. The gelation time of both the gelatin high-Ph (Fig. 1b) and gelatin low-Ph (Fig. 1c) hydrogels decreased with increasing HRP concentration (0.078–0.3125 units/ml) and/or decreasing H_2O_2 concentration (5 mM–0.625 mM). No gelation occurred without the addition of both HRP and H_2O_2 . Using final concentrations of 0.078 units/ml of HRP and 2.5 mM H_2O_2 , the gelation time for 10% (w/v) gelatin-Ph conjugates was 123 ± 5 s for gelatin low-Ph hydrogels and 58 ± 2 s for 10% gelatin high-Ph hydrogels, due to the increased Ph group incorporation into gelatin.

These results demonstrated that we could successfully prepare gelatin gels by cross-linking the introduced Ph groups to porcine gelatin via a peroxidase-catalyzed enzyme reaction. One significant result, which is promising for applications requiring *in situ* gelation, was that we could obtain gelatin hydrogels that formed in less than 1 s or up to 150 s by modifying the concentrations of HRP and H₂O₂ and the content of Ph groups (gelatin high-Ph or low-Ph). A higher concentration of H₂O₂ resulted in a longer gelation time, with a longer time for initiation and completion of the gelation process, a finding that has been reported previously [25–27,35].

When the *in vitro* hemocompatibility of the polymer solutions was studied, gelatin high-Ph and low-Ph at concentrations of 1 mg/ml exhibited little hemolytic activity, less than 4%, similar to that of the PBS negative control (Fig. 1d).

We then examined the rheological properties of the storage modulus (elastic response, G') (left axis) and loss modulus (viscous behavior, G'') (right axis) of rat tail collagen type I gels (control) and gelatin high-Ph (high) and low-Ph (low) hydrogels formed using either 1.25 (Fig. 1e) or 0.078 (Fig. 1f) units/ml of HRP and the indicated concentration of H₂O₂ in step 2. Control rat tail collagen type I gels had a non-tunable G' of $\sim 106 \pm 11$ Pa and non-tunable G'' of $\sim 15 \pm 1$ Pa.

Using 1.25 units/ml of HRP (Fig. 1e, filled symbols), both the gelatin high-Ph and gelatin low-Ph hydrogels showed an increase in G' with an increase in H₂O₂ concentration from 0.31 to 5 or 10 mM, followed by a decrease at higher H₂O₂ concentrations, probably due to deactivation of HRP by the high concentration of H₂O₂; however, the changes were much larger for the gelatin high-Ph hydrogels. The loss modulus (G'') of both the gelatin high-Ph and low-Ph hydrogels was 11–23 Pa and independent of the H₂O₂ concentration (Fig. 1e, nonfilled symbols).

Using 0.078 units/ml of HRP, the same trend of an increase in G' , followed by a decrease with increasing H₂O₂ concentration (probably due to deactivation of HRP by high level of H₂O₂), was seen with the gelatin high-Ph hydrogel, together with a significant decrease in hydrogel stiffness compared to the gel formed using 1.25 units/ml of HRP (Fig. 1e), while little variation was seen with the gelatin low-Ph hydrogel (Fig. 1f, filled symbols). The G'' ranged from 2 to 6 Pa (Fig. 1f, nonfilled symbols).

The dependence on the H₂O₂ concentration (Fig. 1e and f) indicates that, using conjugates with higher phenol content, a higher concentration of H₂O₂ is required to maximize cross-linking. Thus, too low a HRP concentration would result in an incomplete enzyme-mediated reaction and in a decrease in the G' and a slower gelation time (Fig. 1b, c, e and f). The storage modulus of gelatin-Ph hydrogels was sensitive to the degree of cross-linking and could be tuned to levels that are close to those suitable for the growth of blood vessels (<100 Pa)[20].

As a result of the higher crosslinking density in gelatin high-Ph hydrogels than in gelatin low-Ph hydrogels, using a HRP concentration of 0.078 units/ml, the swelling ratio increased with a decrease in Ph content and with an increase in the H₂O₂ concentration (Fig. 1g). Rat tail collagen gels had almost the same swelling properties ($11.3 \pm 3.8\%$) as the gelatin high-

Ph hydrogels ($10.9 \pm 0.2 \sim 12.7 \pm 6.3\%$), regardless of the H_2O_2 concentration used, while the values for the gelatin low-Ph hydrogels ranged from about 10% to about 80%.

Proteolytic degradability of the gelatin-Ph hydrogels could also be tuned by altering the H_2O_2 concentration and the content of Ph groups. Varying the degree of crosslinking using H_2O_2 concentrations from 0.31 to 10 mM resulted in changes in the degradation rates of gelatin-Ph hydrogels. When degraded with collagenase type I, gelatin high-Ph hydrogels with higher crosslinking (i.e. formed at higher H_2O_2 concentrations) showed a slower loss of mass (Fig. 1h; 150 and 300 min digestion) than gelatin low-Ph hydrogels (Fig. 1i; 90 and 150 min digestion). Moreover, both gelatin high-Ph and gelatin low-Ph hydrogels were degraded more slowly than the rat tail collagen hydrogel. After 300 min, only 12.5% of the rat tail collagen remained, while the corresponding values for the gelatin high-Ph hydrogels formed using 0.31, 1.25, and 5 mM H_2O_2 were, respectively, $33.8 \pm 1.5\%$, $84.9 \pm 0.3\%$, and $66.3 \pm 11.1\%$. After 150 min, 47% of the rat tail collagen hydrogel remained, while the corresponding values for the gelatin low-Ph hydrogels were $59.4 \pm 4.5\%$ at 0.31 mM H_2O_2 , increased to $77.4 \pm 0.9\%$ at 1.25 mM H_2O_2 , then decreased to $51.7 \pm 3.8\%$ at 10 mM H_2O_2 .

Injectable biomaterials are designed to fill irregular spaces and therefore, ideally, gelation should not cause significant swelling or shrinkage, as this might compromise the intended functions. As opposed to matching the properties of native blood vessels, the approach taken in this study was to investigate how varying the degree of cross-linking and biodegradation of gelatin-Ph hydrogels influenced vascular density and the overall spatial organization of cells undergoing angiogenesis and vasculogenesis. In all subsequent studies, we used gelatin low-Ph conjugates as our study model and a HRP concentration of 0.078 unit/ml, as gelling occurred in less than 3 min using 0.31–2.5 mM H_2O_2 . This combination is very efficient and convenient for gel formation as an injectable system for surgical and clinical use.

Cross-linking methods to covalently cross-link gelatin hydrogels by adding functional groups to gelatin have been tested. A UV-photocrosslinkable methacrylated gelatin (GelMA) hydrogel [21,22] in an injectable form has been used to deliver cells in applications that require the formation of vascular networks *in vivo* [13,23,36]. The main drawback of this method is the absorption of UV light by the skin (>99%), which results in non-uniform crosslinking of hydrogels [23,37,38]. Visible light is less absorbed by the skin, but more efficient and cytocompatible initiators need to be developed [39]. The possibilities that heat, any residual species produced during polymerization, and the toxicity of the photo-initiator and monomers may damage the surrounding tissues must also be considered. Recently, methods for the enzyme-mediated covalent cross-linking of polymers using HRP, tyrosinase, or transglutaminase have been developed as a versatile system of significant biomedical interest due to the mild gelation process and the ability to modulate the physical and mechanical properties of the gel by varying the reaction parameters [24]. In the present study, regardless of the degree of Ph conjugation or the HRP or H_2O_2 concentration, 10% solutions of gelatin-Ph conjugates formed uniform hydrogels with tunable gelation time, mechanical properties, swelling, and proteolytic degradation. Interestingly, in our system, when we used different hydrogels with a similar G' , in some cases, the proteolytic degradability and swelling properties of the hydrogels could be quite different, i.e. the 10%

gelatin low-Ph hydrogels formed using 0.078 units/ml of HRP and 0.31–2.5 mM H₂O₂, and we therefore examined cell behavior on these different gels.

3.3. Influence on the behavior of human cord blood-derived ECFCs and white adipose-derived MSCs

Gelatin-Ph hydrogels have several promising attributes for use with different cell types (L929 fibrosarcoma cell lines [28], neural stem cells [27], the ATDC-5 chondrocyte cell line [40], and human mesenchymal stem cells [25,26,29]), but whether they can support human endothelial progenitor cells to create vascular networks *in vitro* or produce a robust vasculature *in vivo* has not been studied. We therefore evaluated the ability of ECFCs and MSCs to attach to, and proliferate and spread on/within, gelatin hydrogels with almost the same values for the storage modulus ($G' \sim 153 \pm 22 \sim 191 \pm 17$ Pa) and loss modulus ($G'' \sim 4$ Pa) (Fig. 1f), but with different susceptibility to proteolytic degradation as a result of using different H₂O₂ concentrations (0.31–2.5 mM).

First, we examined what H₂O₂ concentrations used to form hydrogels were compatible with *in vitro* cell viability and found that attachment (Fig. 2a) and viability (Fig. 2b) of both ECFCs and MSCs cultured on hydrogels was not markedly affected after 6 h of culture on gelatin low-Ph hydrogels crosslinked with a wide range of H₂O₂ concentrations (0–2.5 mM). Culture for 24 h on these same gels had little effect on the proliferation of MSCs (Fig. 2c) or the survival of both cell types (Fig. 2d), but resulted in a marked decrease in ECFC proliferation. In terms of spreading, increasing the H₂O₂ concentration from 0.31 mM to 2.5 mM, i.e. decreasing the proteolytic degradability, decreased the ability of both ECFCs and MSCs to spread in 24 h (Fig. 2e).

An injectable gelatin-hydroxyphenylpropionic acid (Gtn-HPA) hydrogel scaffold has been shown to have tunable stiffness, making it possible to control the proliferation and differentiation of human bone marrow-isolated MSCs in a 2D (plated on the hydrogel) or 3D (encapsulated in the gel) cell culture environment [25,26]. In 2-D cell culture systems, MSCs grown on a softer hydrogel ($G' \sim 600$ Pa) were found to express higher levels of neurogenic protein markers than those on a stiffer hydrogel ($G' \sim 12,800$ Pa), while those grown on the stiffer hydrogel showed higher upregulation of myogenic proteins than those on the softer hydrogel [26]. Due to the inherent problems of 3-D cell culture systems, such as poor transportation of nutrients and low degradability of hydrogels as a result of increasing stiffness, the storage modulus of hydrogels used to study the differentiation of these MSCs in 3D hydrogels was limited to no more than 1000 Pa [19]. These results show that the stiffness and degradation properties of hydrogels play an important role in stimulating the differentiation of encapsulated stem cells. In our previous studies [13,23], we found that increasing the degree of GelMA polymerization results in an increase in the storage modulus (20 Pa using 15 s of UV exposure, 200 Pa using 30 s, and 1000 Pa using 45 s), accompanied by a decrease in degradation that would limit the ability of both ECFCs and MSCs to spread inside GelMA. Addition of exogenous collagenase partially restores the migratory ability of cells grown inside gels polymerized using 30–45 s of UV, suggesting that cell spreading and motility are mainly inhibited as a result of decreased proteolytic degradability. We also found that GelMA constructs that are photocrosslinked for 15 s ($G' \sim$

20 Pa) form an extensive network of ECFC-lined microvessels after 7 days *in vivo*, while those that are photo-crosslinked for 45 s ($G' \sim 1000$ Pa) do not form luminal structures, and ECFCs are predominantly distributed as individual cells. These GelMA hydrogels photo-crosslinked using different UV exposure times (15, 35, and 45 s) possess the same density of RGD integrin binding sites, but those crosslinked using longer UV exposure have high mechanical properties and low proteolytic degradability, resulting in less angiogenesis and vasculogenesis both *in vitro* and *in vivo* [13,23]. Other authors [41] fabricated synthetic biomimetic hydrogels with different degrees of rigidity containing integrin binding sites (RGDS) and matrix metalloproteinase-sensitive substrates (GGGPQGYIWGQGK, MMPs) to mimic natural extracellular matrices that allow the rapid formation of a stable vascular network *in vitro* and the ingrowth of host vasculature *in vivo*, and the results showed that those with an intermediate rigidity resulted in the best angiogenic responses of endothelial cells co-cultured with fibroblasts. However, these authors also demonstrated that the mechanical properties of hydrogels may not be the sole determinant of angiogenic potential, since other parameters, such as the density of cell adhesion sequences or degradable sites, might be involved [41,42]. Moreover, the mechanical and biodegradation properties of photo-polymerized hydrogels are not easy to control, as fast curing rates, used to avoid long exposure to UV, make it difficult to control mechanical and degradation properties. In contrast, in our gelatin-Ph hydrogel system, the physical and chemical properties of gelatin-Ph hydrogels can be readily controlled by controlling the concentrations of reagents, such as H_2O_2 and HRP, and the amount of Ph conjugated to gelatin. Over the H_2O_2 concentration range tested, all gelatin-Ph hydrogels used for cell studies were relatively soft and had similar stiffness ($G' \sim 153 \pm 22 \sim 191 \pm 17$ Pa), but degradation decreased with increasing H_2O_2 concentration (Fig. 1f, g and i), indicating that, in hydrogels with the same G' , proteolytic degradation regulates cell spreading and proliferation behavior.

3.4. Enzymatic and oxidative stress resistance of cells encapsulated in hydrogels

In this section, it should be noted that HRP/ H_2O_2 were first used to generate the hydrogel, and then were also used as stress agents. The viability of NIH-3T3 cells encapsulated within, or grown under gels was measured to assess the effects of gelatin-Ph hydrogels on the exogenous enzymatic or oxidative stress on cells generated using HRP or H_2O_2 .

Using gelatin high-Ph hydrogels cross-linked using 0.078 units/ml of HRP and 1.25 mM H_2O_2 , cells embedded within, or grown on, gels were incubated in medium containing different concentrations of HRP or H_2O_2 for 24 h. When enzymatic stress was applied by the addition of 0.3125 units/ml to 10 units/ml HRP to the culture medium for 24 h, the viability of the encapsulated cells remained higher than 80% (Fig. 3a, red circles), but the viability of cells grown on the gel fell to around 60% using 5 units/ml of HRP and 29.6% using 10 units/ml (Fig. 3a, black squares). When H_2O_2 was added to the culture medium at concentrations from 3.125 to 100 μ M, the viability of the encapsulated cells remained greater than 90% at H_2O_2 concentrations up to 25 μ M, then fell to $52.8 \pm 3.3\%$ at 100 μ M H_2O_2 (Fig. 3b, red circles). In contrast, the viability of cells grown on gels was 72.4% at 6.25 μ M H_2O_2 and fell abruptly to 15% at H_2O_2 concentrations 25 μ M (Fig. 3b, black squares). The high tolerance of the encapsulated cells to HRP/ H_2O_2 stress prompted us to evaluate the factors responsible. One possibility was that a proportion of the tyramine

moieties on gelatin-Ph might remain uncoupled after crosslinking and help protect encapsulated cells from oxidative stress by scavenging radicals generated from H_2O_2 or by increasing the activity of cellular enzymes that help break down H_2O_2 . We tested this hypothesis by incubating cells on gelatin high-Ph gels for 24 h with 12.5 μM H_2O_2 in the presence of soluble gelatin high-Ph conjugates to determine if the cytotoxicity effects of oxidative stress were ameliorated by soluble conjugates, but found that this was not the case (Fig. 3b, purple triangles). In contrast, culturing cells under gelatin high-Ph hydrogels resulted in cell numbers at 3.125 mM H_2O_2 that were as high as in the absence of H_2O_2 and, in fact, increased to 125–150% at higher H_2O_2 concentrations (Fig. 3b, blue inverted triangles). It is therefore likely that the gelatin-Ph hydrogels, and not gelatin-Ph conjugates, are responsible for the increased oxidative stress resistance observed in cells encapsulated within or grown under these gels. Such increased cytocompatibility may have resulted from the barrier created by the hydrogel, which slows down or obstructs the diffusion of H_2O_2 and HRP from the medium to the cultured cells. Alternatively, since the cells were transiently exposed to H_2O_2 during their encapsulation within or under gelatin-Ph hydrogels, the higher oxidative stress resistance of cells grown inside or under the hydrogel might have been due to an altered state of the cells caused by preconditioning, exposure to the HRP/ H_2O_2 used to crosslink gelatin-Ph hydrogels could have a preconditioning effect on cells embedded inside gels or cells that might increase their resistance to exogenous enzymatic and oxidative stress, as reported by Spector's group, who used HRP and H_2O_2 to crosslink hydroxyphenylpropionic acid (Gtn-HPA) hydrogels[27]. In the lesion site after tissue injury, a biomaterial and associated crosslinking chemistry that precondition cells and increase their resistance to oxidative stress would be beneficial.

3.5. In vivo formation of human vascular networks

We then examined whether changing the H_2O_2 concentration used to crosslink the gel modulated vascular morphogenesis *in vivo*. To test this, we injected 1.2×10^6 MSCs and 0.8×10^6 ECFCs in 250 μl of porcine gelatin low-Ph hydrogel crosslinked using 0.078 units/ml of HRP and 0.31, 0.63, 1.25, or 2.5 mM H_2O_2 into nude mice, then, after 7 days, explanted the constructs and examined them for the presence of vascular structures. Hematoxylin and eosin (H&E) staining revealed that the extent of vascular network formation was indeed affected by the H_2O_2 concentration, as the number of lumens inside the construct decreased with increasing H_2O_2 concentration (Fig. 4a). Quantitative evaluation of the explants revealed a significant reduction in the total number of perfused blood vessels at H_2O_2 concentrations of 1.25 mM (35.93 ± 12.1 lumens/ mm^2) and 2.5 mM (30.1 ± 14.4 lumens/ mm^2) compared to 0.31 mM (70.37 ± 26 lumens/ mm^2) and 0.63 mM (73.13 ± 43.8 lumens/ mm^2) (Fig. 4d). In addition, the H_2O_2 concentration affected the morphology of the newly formed mature human microvessels, identified by staining for hCD31 (Fig. 4b), which were completely covered by αSMA -positive perivascular cells (Fig. 4c). The mean areas of the human lumens in constructs crosslinked using all four H_2O_2 concentrations were in the range of $218 \pm 132.8 \mu\text{m}^2$ to $255.1 \pm 123.7 \mu\text{m}^2$ (Fig. 4e). Using 0.31 or 0.63 mM H_2O_2 , most human microvessels had distinguishable lumens, whereas, in the 1.25 and 2.5 mM H_2O_2 groups, fewer well-formed lumens were seen and hCD31 + ECFCs were predominantly found as individual cells, suggesting a lack of cell motility (Fig. 4b and c). These results demonstrate that the decreased proteolytic degradation of more highly

crosslinked gels formed at a higher H₂O₂ concentration can modulate the overall vascular density, but not the vascular area. The percentage of human lumens inside porcine gelatin-Ph hydrogels generated using 0.31–1.25 mM H₂O₂ was approximately ~50%, but fell to ~20% using 2.5 mM H₂O₂ (Fig. 4f).

3.6. Extraction of murine gelatin and synthesis of murine gelatin-Ph hydrogels

Although naturally-derived polymer and acellular matrices from different species can provide cell attachment, proliferation, and differentiation signals and be degraded into physiologically tolerable compounds, there are concerns about potential risk of pathogen transmission and provocation of undesirable inflammatory and immunological reactions, leading to failure of regenerated or transplanted tissues and organs [43,44]. However, an autologous extracellular matrix scaffold should be a safe and reliable biomaterial candidate. Extracellular matrix scaffolds have been extensively used in tissue engineering and regenerative medicine to eliminate negative host responses and lead to optimal tissue regeneration [43,45–48]. In our study, we evaluated the use of autologous murine gelatin extracted from the dermal skin conjugated with Ph groups for potential use in vascular network tissue engineering. Acid treatment causes swelling of skin gelatin and removal of non-collagenous protein, and autoclaving (121 °C for 30 min) breaks down collagen to produce smaller gelatin molecules. The SDS–PAGE pattern of murine epidermal gelatin extracted in this way was found to contain more peptides and a lower proportion of high molecular weight fractions than porcine gelatin (Fig. 5a). In terms of the purity of murine gelatin prepared using our method, the hydroxyproline (hyp) content was 0.135 mg/mg, almost the same as that of 0.14 mg/mg for commercial porcine gelatin (Sigma–Aldrich). In terms of scalability of the process, the average yield of murine gelatin extracted from 100, 500, or 1000 mg of freeze-dried mouse epidermal skin was around 30.12 ± 4.8 mg/g. Fig. 5b lists the amino acid compositions of gelatin extracted from porcine or murine skin expressed as amino acid residues per 1000 total amino acid residues. The murine gelatin had a higher content of glycine than porcine gelatin and slightly lower contents of hydroxyproline, proline, alanine, and glutamic acid. The proline plus hydroxyproline content of murine gelatin was 206.8 residues/1000 residues (20.6%), much higher than those of gelatins from marine sources, such as Nile tilapia (17.6%) [49] and bigeye snapper skin (18.7%) [50], but slightly lower than that of commercial porcine gelatin (25.6%, Sigma Aldrich). Fig. 5c shows the zeta potential as a function of pH of porcine and murine gelatin in distilled water; when the zeta potential is close to zero, particles are formed and this results in an unstable suspension. The pI_{IEP} (pH at which the zeta potential is zero) in distilled water was $\gg 8$ for porcine gelatin and 8 for murine gelatin. Gelatin can increase cell function, such as adhesion, spreading, proliferation, and migration, and has thus been extensively used to enhance cell and material interactions for both *in vitro* and *in vivo* applications. To investigate its effect on cell proliferation, *in vitro* studies were carried out using ECFCs, and numbers of viable cells were assessed by the MTS assay. As shown in Figs. 5d and S3, compared to porcine gelatin as the control coating material, cell numbers were slightly higher after 2 days of culture on plates coated with rat tail collagen type I or murine gelatin.

In order to conjugate Ph groups to murine gelatin, we measured the zeta potential of murine gelatin dissolved in 50 mM MES buffer as a function of pH. As shown in Fig. 5e, the zeta

potential was $+13.56 \pm 0.5$ mV at pH 4, 1.16 ± 0.3 mV at pH 6, and -2.6 ± 0.96 mV at pH 8. Thus, the pI_{IEP} of murine gelatin in 50 mM MES was around 6.5. The murine gelatin-Ph conjugate was formed by reacting 2 g of murine gelatin in 100 ml of 50 mM MES, pH 6, with 0.735 g of EDC and 0.221 g of NHS at 27 °C for 12 h. The ¹H NMR spectrum of the conjugate showed peaks at chemical shifts (δ) of 6.8 ppm and 7.1 ppm that were not seen in unmodified murine gelatin (Fig. 5f), indicating successful conjugation of tyramine to carboxyl groups. The total Ph content incorporated under the preparation conditions was 2.96×10^{-2} g per g of gelatin (murine gelatin-Ph). Macroscopic images showed there was no difference in the opacity of the gels formed with increasing H₂O₂ concentrations (1.25–5 mM) and 0.078 units/ml of HRP (Fig. 5g). The hemocompatibility of the murine gelatin and the murine gelatin-Ph conjugate at a concentration of 10 mg/ml was also studied. As shown in Fig. 5h, murine gelatin caused about 10% hemolysis and murine gelatin-Ph less than 1% hemolysis, a result similar to that seen for the negative control PBS. We have therefore developed a method for the successful production of an autologous murine gelatin-Ph conjugate, aqueous solutions of which are gellable by a peroxidase-catalyzed enzyme reaction to yield murine gelatin-Ph hydrogels with tunable gelation time, mechanical properties, and proteolytic degradability.

3.7. Comparison with other natural hydrogels

Timing is an intrinsic constraint in most tissue engineering concepts. Typically, constructs greater than approximately 1 cm in thickness cannot rely solely on vascular ingrowth from the host to remain viable *in vivo*, as the blood supply to the center is inadequate and necrosis occurs, so the development of three-dimensional biomaterials that can improve and speed up vascularization of regenerative tissues/constructs within 7 days is an important factor. One major hurdle in the clinical application of large engineered tissues and organs has been securing the blood supply to the graft at the time of implantation [1,4]. One appealing strategy among the many being currently explored to overcome this problem has been to develop scaffolds that not only build a microcirculation network within the engineered scaffold itself, but also result in ingrowth of host blood vessels within a week. We therefore compared the extent of vascular network formation in porcine gelatin low-Ph and murine gelatin-Ph constructs to that seen with other injectable hydrogels commonly used in this field of research, namely rat tail collagen type-I and Matrigel. Rat tail collagen type-I and Matrigel cell-laden constructs were formed by adding human ECFCs and MSCs to a solution of collagen type-I (3 mg/ml) or Matrigel, then 250 μ l (2×10^6 cells; 2:3 ECFC:MSC ratio) was injected subcutaneously into nude mice before gelation, while, for the murine gelatin-Ph or porcine gelatin low-Ph cell-laden constructs, 250 μ l of Ca/Mg-free DPBS containing 10% (w/v) gelatin-Ph conjugates, 0.078 units/ml of HRP, 0.63 mM H₂O₂, and human ECFCs and MSCs (2×10^6 cells; 2:3 ECFC:MSC ratio) was injected subcutaneously, then, after 7 days *in vivo*, the constructs were recovered and evaluated. As shown in Fig. 6, as expected from our previous results [7,18,30,32], both rat tail type-I collagen and Matrigel were suitable for ECFC/MSC-mediated vascular network formation. However, quantitative analysis of H&E-stained sections taken from the explanted constructs at day 7 revealed that vascular network formation was significantly higher in the porcine gelatin low-Ph hydrogel (73.13 ± 43.8 lumens/mm²) and murine gelatin-Ph hydrogel (59.99 ± 22.8 lumens/mm²) than in those formed with rat tail collagen type-I (33.61 ± 15.9

lumens/mm²) or Matrigel (18.60 ± 10.95 lumens/mm²) (Fig. 6d). The human origin of the microvessels was confirmed immunohistochemically by staining for hCD31 (Fig. 6b) and hCD31 plus α SMA (Fig. 6c). The lumens in the porcine and murine gelatin-Ph constructs were about the same size as those in rat tail collagen type-I and Matrigel (Fig. 6e). In the rat tail collagen type I hydrogels and the murine and porcine gelatin low-Ph hydrogels, most human microvessels had distinguishable lumens (Fig. 6a–c), while, in the Matrigel group, well-formed lumens alternated with non-luminal ECFC-lined cord structures (Fig. 6b and c) and non-assembled individual ECFCs. In particular, in the murine and porcine gelatin low-Ph hydrogels, a higher percentage of the lumens were human, indicating that the biocompatibility of our porcine and murine gelatin-Ph constructs was suitable not only for angiogenesis, but also for vasculogenesis.

To determine the mechanical strength provided by hydrogels for the encapsulated cells, we examined the rheological properties of G' (left axis) and G'' (right axis) of rat tail collagen type I gels (control), Matrigel, and porcine gelatin low-Ph hydrogels formed using 0.078 units/ml of HRP and the indicated concentration of H₂O₂ in step 2 after one day of swelling in PBS (Fig. S4a). The rat tail collagen gels and Matrigel had, respectively, a non-tunable G' of $\sim 105.8 \pm 10.6$ Pa and 39.1 ± 2.7 Pa and a non-tunable G'' of $\sim 15.3 \pm 0.7$ and 2.7 ± 0.1 Pa. In contrast, while the G'' value after swelling in PBS of gelatin low-Ph hydrogels formed using 0.31–2.5 mM H₂O₂ was 1–7 Pa and independent of the H₂O₂ concentration, they showed an increase in G' with an increase in H₂O₂ concentration from 0.31 to 1.25 mM (70.6 ± 5 Pa at 0.31 mM; 135.5 ± 5.5 Pa at 1.25 mM), followed by a decrease at 2.5 mM (117.9 ± 3.8 Pa) due to HRP deactivation by the high concentration of H₂O₂. The *in vivo* biodegradation of the hydrogels was evaluated by the mass of hydrogel remaining after one week of subcutaneous implantation in mice (Fig. S4b). Varying the degree of crosslinking using H₂O₂ concentrations from 0.31 to 2.5 mM resulted in changes in the *in vivo* degradation rate of porcine gelatin low-Ph hydrogels. After one week of implantation, those with higher crosslinking (i.e. formed at 1.25 mM H₂O₂) showed less loss of mass (residue mass $\sim 6.5 \pm 2.6\%$) than those with lower crosslinking (i.e. formed at 0.31 mM H₂O₂) (residue mass $\sim 2.6 \pm 0.1\%$). Moreover, those formed using 0.31–2.5 mM H₂O₂ were degraded more rapidly than rat tail collagen type I gels (residue mass $\sim 8.7 \pm 3.9\%$) or Matrigel (residue mass $\sim 39.9 \pm 3.4\%$).

To summarize the above results, our cell-laden porcine gelatin low-Ph constructs formed using 0.31 or 0.63 mM H₂O₂ and our murine gelatin-Ph constructs formed using 0.63 mM H₂O₂ had significant higher vascular densities than those formed using the same cells and other common hydrogels, i.e. Matrigel or rat tail collagen type I. These 10% gelatin low-Ph hydrogels enzymatically crosslinked using different H₂O₂ concentrations contained the same density of the cell adhesion sequence, RGDS, and matrix metalloproteinase-degradable sites, but those crosslinked using less H₂O₂ (i.e. 0.31 or 0.63 mM) had a lower G' (75 or 100 Pa) and higher proteolytic degradability and resulted in the formation of mature human vessels within cell-laden gels. Based on our results, we suggest that hydrogels suitable for supporting ECFC/MSCs to form a vasculature and anastomosis with the host vasculature require certain amounts of cell adhesion sequence and matrix metalloproteinase-

degradable sites, intermediate proteolytic degradability at storage modulus G' around 75–100 Pa.

The suitability of injectable and enzymatically crosslinked gelatin low-Ph hydrogels with different gelation times, mechanical properties, swelling behaviors, and proteolytic degradability was evaluated. Increasing the concentration of H_2O_2 used, which controls the crosslinking density between gelatin-Ph conjugates resulting in hydrogels with a higher storage modulus and lower proteolytic degradability, did not affect the viability and attachment of ECFCs and MSCs grown on gels, but resulted in less spreading and proliferation of ECFCs. In addition, we also demonstrated that vascularization can be modulated by adjusting the degree of polymerization of the enzymatically crosslinked hydrogels by altering the H_2O_2 concentration used *in vivo*. This ability to modulate vascular density might help in bioengineering tissues with different vascular requirements. In our case, even though porcine gelatin low-Ph hydrogels with a wide range of mechanical properties could be formed, only a few (those produced using H_2O_2 concentrations lower than 0.63 mM) were found to be compatible with ECFC/MS cell motility and spreading and vascular morphogenesis. In addition, autologous murine gelatin-Ph hydrogels were synthesized and shown to be suitable for use in vascularized tissue engineering and regenerative medicine by eliminating negative host responses and leading to optimal tissue regeneration. Numerous studies [6,7,10,14,15,17–19] have shown that several natural hydrogels can generate permissive environments for vascular morphogenesis. However, it is the ability to modulate vascularization that makes both porcine and murine gelatin-Ph hydrogels more suitable for applications that require the formation of functional vascular beds, including the engineering of complex tissues. Our results show that gelatin-Ph hydrogels containing ECFCs and MSCs are able to support ECFC activity inside the hydrogel, promoting vascularization in the cell-laden construct.

4. Conclusions

Difficulties have been experienced in engineering thick, complex, highly vascularized tissues and organs, primarily due to the lack of an efficient exchange system for delivery of nutrients and oxygen and removal of waste through a functional vasculature. In this study, we have demonstrated the suitability of injectable and enzymatically crosslinkable gelatin-Ph hydrogels for use in supporting human progenitor cell-based formation of 3D vascular networks *in vitro* and *in vivo*. Using porcine gelatin-Ph hydrogels as the embedding scaffold, we showed that 3D co-implants of human cord blood-derived ECFCs and white adipose tissue-derived MSCs generate extensive human capillary-like networks that form functional anastomoses with the existing vasculature on implantation into immunodeficient mice. We also demonstrated that the mechanical and biodegradation properties of gelatin-Ph hydrogels can be controlled by altering the degree of crosslinking, and that this can be used to tune the extent of vascular network formation *in vivo*. In addition to their role as a supporting scaffold, gelatin-Ph hydrogels act as a barrier to the effects of exogenous enzymatic and oxidative stress on encapsulated cells and thus increases cell viability. In addition, we report a method for preparing autologous extracellular matrix scaffolds, in our case, murine gelatin-Ph hydrogels. The use of cell-laden murine gelatin-Ph hydrogels as implants in immunodeficient mice resulted in the rapid formation of functional anastomoses between the

bioengineered human vascular network and the mouse vasculature within one week of implantation. Based on these data, we propose the use of gelatin-Ph hydrogels in regenerative engineering applications, including the engineering of 3D thick tissues and organs that require an adequate vascular supply to guarantee their survival and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1 and 3–6, are difficult to interpret in black and white. The full color images can be found in the on-line version, at <http://dx.doi.org/10.1016/j.act-bio.2015.02.024>.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2015.02.024>.

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Fig. 1. Enzymatic polymerization of porcine gelatin-Ph hydrogels with different contents of phenolic hydroxyl (Ph) groups: (a) ^1H NMR spectra of 0.5% (w/v) solutions of unmodified gelatin and gelatin high-Ph and gelatin low-Ph conjugates in D_2O . (b and c) Dependence of gelation time of 10% (w/v) (b) gelatin high-Ph and (c) gelatin low-Ph conjugates crosslinked using the indicated concentrations of HRP (symbols) and H_2O_2 (x axis). (d) Hemolysis test of 1% (w/v) unmodified gelatin and gelatin high-Ph and gelatin low-Ph conjugates. Normal saline was used as the negative control and distilled water as the positive control. (e and f) Mechanical properties G' (left axis) and G'' (right axis) of 10% (w/v) gelatin high-Ph (black squares) and low-Ph (red circles) hydrogels formed using 1.25 (e) or 0.078 (f) units/ml of HRP and the indicated concentration of H_2O_2 . The blue triangles are results for rat tail collagen hydrogels (g) Swelling ratio of the gels in (e and f) (h and i) Degradation of 10% (w/v) gelatin high-Ph (h) or gelatin low-Ph (i) hydrogels after incubation with collagenase type I for 150 or 300 min (h) or 90 or 150 min (i). Degradation of rat tail collagen gels are shown on the right. (b–i) Data in (b–i) are the mean $\pm$ standard deviation for 3–5 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared to rat tail

collagen gels at the same time point. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Fig. 2.

Effects of the degree of crosslinking of porcine gelatin low-Ph hydrogels on various functions of ECFCs and MSCs. Attachment (a) and number of viable cells (b) after 6 h of culture, and proliferation (c), number of viable cells (d), and (e) spreading area after 24 h of culture of ECFCs (white bars) or MSCs (hatched bars) on gelatin low-Ph hydrogels cross-linked using 0.078 units/ml of HRP and the indicated H₂O₂ concentration ($n = 3-5$). Porcine gelatin-coated culture plates were used as control. Results are expressed as a percentage of the value for the number of cells on gelatin-coated dishes after 4 h of culture (a). The results are expressed as a percentage of the value for the viable number of cells on gels after 4 h (b) and 24 h (c and d) of culture. Data are the mean $\pm$ standard deviation for 3-5 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the porcine gelatin-coated dish (control). # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared to the gelatin-Ph hydrogels crosslinked using 0.31 mM H₂O₂.

Fig. 3.

Porcine gelatin high-Ph hydrogels increase the enzymatic and oxidative stress resistance of encapsulated NIH-3T3 fibroblasts. Number of viable NIH3T3 fibroblasts encapsulated within (red circles), or grown on (black squares), gelatin high-Ph hydrogels after incubation for 24 h in culture medium containing different concentrations of (a) HRP or H₂O₂ (b). Results are expressed as a percentage of the number of cells after 24 h culture in the absence of HRP and H₂O₂ and are the mean $\pm$ standard deviation for 3–5 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to cells grown on gels using the same concentrations of HRP or H₂O₂. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Enzymatic modulation of vascular network bioengineering. Cell/porcine gelatin low-Ph conjugate mixtures were subcutaneously injected into nude mice and enzymatically polymerized using 0.078 units/ml of HRP and the indicated concentration of H_2O_2 , then the constructs were removed and evaluated after 7 days *in vivo*. (a) Representative H&E-stained sections of constructs with different degrees of crosslinking of gelatin low-Ph hydrogels at low magnification (top panels) and high magnification (bottom panels). The insets in the top panels show macroscopic views of the explants (scale bar 2 mm). In the bottom panels, the black arrowheads indicate perfused lumens and the blue arrowheads non-perfused lumens. (b) Human CD31 + ECFCs identified by immunohistochemistry. The red arrowheads indicate areas with perfused lumens lined by CD31 + ECFCs and the green arrowhead areas

with individual CD31 + ECFCs. (c) Representative images of sections stained using Alexa 488-conjugated-anti-CD31 antibodies (green) to label human ECFC-lined vessels and Alexa 594-conjugated anti- α SMA antibodies (red) to label perivascular cells. Anti-CD31 antibodies do not bind to murine vessels. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). (d–f) The extent of human vascular network formation was quantified by counting luminal structures containing erythrocytes: (d) microvessel density, (e) size distribution of the luminal cross-sectional area, and (f) percentage of the total blood vessels that are human (CD31-expressing). Data are the mean $\pm$ standard deviation for 4–6 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared to hydrogels crosslinked with 0.31 mM H₂O₂. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Extraction of murine gelatin and characterization of murine gelatin-Ph hydrogels. (a–d): Murine gelatin (a) SDS–PAGE patterns of standard protein markers (outer lanes), porcine gelatin (Sigma–Aldrich), and murine gelatin. (b) Amino acid composition of murine gelatin and porcine skin gelatin (Sigma–Aldrich). (c) ζ potential measurement of murine gelatin and porcine skin gelatin (Sigma–Aldrich) in double-distilled water at different pHs. (d) Proliferation of ECFCs after 2 days of culture on polystyrene plates coated with porcine gelatin, set as 100%, rat tail collagen, or murine gelatin. Data are presented as the mean $\pm$ standard deviation for 3–5 independent experiments; no significant differences were seen compared to porcine gelatin-coated cell culture plates. (e–h) Synthesis of murine gelatin-Ph hydrogels: (e) ζ potential measurements of murine gelatin (red circles) and porcine gelatin (Sigma–Aldrich) (black squares) in MES buffers at different pHs, (f) ^1H NMR spectra of 1% (w/v) murine gelatin and murine gelatin-Ph dissolved in D_2O , (g) Photographs of 10% (w/v) murine gelatin-Ph solutions at 3 min after the addition of 0.63 units/ml of HRP and the indicated concentration of H_2O_2 , (h) Hemolysis test of murine gelatin and gelatin-Ph hydrogels. Data are the mean $\pm$ standard deviation for 3–5 independent experiments. (For

interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Comparative evaluation of Matrigel, rat tail collagen type-I, murine gelatin-Ph, and porcine gelatin low-Ph hydrogels. Human ECFCs and MSCs (total 2×10^6 cells; 2:3 ECFC:MSC ratio) were resuspended in a solution of Matrigel, rat tail collagen type-I, murine gelatin-Ph or porcine gelatin low-Ph conjugates and subcutaneously injected into nude mice (volume: 250 μ l); murine gelatin-Ph and porcine gelatin low-Ph implants were polymerized using 0.078 units/ml of HRP and 0.63 mM H₂O₂. Constructs were evaluated after 7 days *in vivo*. (a) Representative H&E-stained sections from each group showing visible erythrocyte-filled microvessels (scale bar 200 μ m in the top panels and 40 μ m in the bottom panels). The insets in the top panels are macroscopic views of the explants (scale bars 2 mm). In the bottom panels, the black arrowheads indicate perfused lumens. (b) Human hCD31 + lumens

identified by immunohistochemistry in each group. The red arrowheads indicate areas with perfused lumens lined by hCD31 + ECFCs, the yellow arrowheads an area with CD31 + ECFCs forming a non-luminal cord-like structure, and the green arrowheads areas with individual hCD31 + ECFCs. (c) Representative images of sections stained with Alexa 488-conjugated-anti-CD31 antibodies (green) to label human ECFC-lined vessels and Alexa 594-conjugated anti- α SMA antibodies (red) to label perivascular cells. Nuclei were stained with DAPI. (d) Microvessel density determined at day 7 by counting luminal structures containing erythrocytes. (e and f) Microvascular lumen area (e) and percentage of human CD31-expressing blood vessels (f). Data are the mean $\pm$ standard deviation for 4–6 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ compared to rat tail collagen gels. # $p < 0.05$ and ### $p < 0.01$ compared to porcine gelatin-Ph hydrogels crosslinked with 0.63 mM H₂O₂. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)